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PRINCIPAL INVESTIGATOR: Jennifer L. Ariazi
Doctor Michael Gould

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, Wisconsin 53706-1490

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6. AUTHOR(S)
Jennifer L. Ariazi
Doctor Michael Gould

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wisconsin
Madison, Wisconsin 53706-1490

E-Mail: jariazi@students.wisc.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Despite intense epidemiological studies of mature women, ionizing radiation is the only agent commonly accepted to cause breast cancer. The immature breast is more susceptible than the mature breast to the carcinogenic effects of ionizing radiation; perhaps the same is true of chemicals. This project's goal is to determine whether immature rat mammary epithelial cells (RMECs) are more susceptible to the mutagenic effects of N-nitroso-N-methylurea (NMU) and begin to explore reasons for that susceptibility. Work reported last year revealed that immature RMECs are indeed more susceptible than mature RMECs to the mutagenic effects of NMU; results hinted that immature RMECs were deficient in methylguanine methyltransferase (MGMT) activity relative to mature RMECs. This year, an assay was developed to measure MGMT activity. RMEC MGMT activity is much lower than MGMT activity in liver, which displays no age-related difference. In contrast, immature RMECs have 2/5 less MGMT activity than mature RMECs ($p = 0.0127$). This DNA repair deficiency in immature RMECs could contribute to age-differential mutagenesis. These results warrant further studies of age-related DNA repair differences and suggest that epidemiological studies of adult women should focus on progression, while efforts to identify initiating agents would be better directed at immature girls.

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INTRODUCTION

The science of epidemiology, which has been very successful in elucidating a number of environmental chemicals with causative roles in human cancers, has found only one environmental agent that causes breast cancer: ionizing radiation. It is known from studies of women irradiated at the atomic bombings of Hiroshima and Nagasaki or medically for the treatment conditions such as Hodgkin's disease, that the immature breast is more susceptible than the mature breast to the carcinogenic effects of ionizing radiation (1, 2). Perhaps no chemical breast carcinogens have been identified because the wrong cohorts – adult women rather than young girls – are being examined. The work presented here was undertaken to determine whether the immature breast is also more susceptible than the mature breast to the effects of chemical carcinogens. Specifically, the goal of this project is to determine whether immature rat mammary epithelial cells (RMECs) are more susceptible than mature RMECs to the mutagenic effects of N-nitroso-N-methylurea (NMU), and to begin to dissect the mechanism of this increased susceptibility. Already it has been determined that immature RMECs harbor more persistent mutations than mature RMECs following NMU treatment. Interesting differences in DNA integrity shortly following NMU treatment have pointed to age-related DNA repair discrepancies not due to apoptosis. Work conducted during this reporting period indicates that there are age-related differences in the activity of methylguanine methyltransferase (MGMT), the primary enzyme responsible for the repair of O^6-methylguanine (O^6-meG), the main promutagenic lesion introduced by NMU treatment. Taken together, these data indicate that the immature RMEC is more susceptible than the mature RMEC to the mutagenic effects of NMU due at least in part to deficiencies in DNA repair activity. This conclusion is consistent with the hypothesis that is the immature human breast that is at the greatest risk for breast cancer initiation. The search for chemical breast carcinogens, then, should focus on exposures not of women as cancer is detected, but young girls. Additionally, these results suggest age-related differences in DNA repair could be a fruitful line of research for the understanding and prevention of at least breast cancer.

BODY

Demonstrate that NMU treatment *in vitro* recapitulates *in vivo* survival phenomenon

No progress has been made in demonstrating that NMU treatment *in vitro* recapitulates the *in vivo* survival phenomenon.

Long-term persisting mutations in RMECs of immature and mature rats

As reported last year, it has been demonstrated that immature RMECs harbor statistically significantly greater levels of persistent mutations than mature RMECs. Verified cored mutant plaques are stored at -80° C awaiting sequence analysis.

Short-term mutations and their repair in RMECs from immature and mature rats

As reported last year, comet assays revealed an age-differential effect beginning two hours following NMU treatment. Immature, but not mature, RMECs displayed increased tail moments, indicative of greater levels of single strand DNA breaks and/or alkali-labile sites, such as AP sites. Apoptosis was not responsible for these differences.

Mechanism of removal of O^6-methylguanine, a known cytotoxic lesion

As reported last year, comet assays of immature and mature RMECs pretreated with benzylguanine, which efficiently inhibits methylguanine methyltransferase (MGMT), indicated that the tail moment difference was due to MGMT activity in the mature RMECs.

Work in this reporting period focused on the methylguanine methyltransferase activity assay. While the original proposal called for the use of an assay that measured the transfer of a tritiated methyl group from probe DNA to MGMT, another methodology using fluorescently-labeled DNA probes was subsequently reported (3). This assay was desirable for numerous reasons. First, it eliminated the need for radioactivity and its concomitant difficulties. Second, the methodology was simpler (oligo probes could be purchased rather than preparing a tritiated probe in-house, requiring special facilities and certifications). Third, it is readily adaptable for the

measurement of additional relevant DNA repair enzymes, including methylpurine DNA glycosylase, 8-oxoguanine DNA glycosylase, and abasic endonuclease (4).

This reporting period was consumed by optimizing and performing the MGMT activity assays. The premise of the assay is this (Figure 1; all figures are in Appendix 1): An 18-bp hex-labeled oligo with a methylguanine residue within a PvuII restriction enzyme recognition sequence is mixed with lysates from immature and mature RMECs (or any other tissue). After a two-hour incubation, the oilgo probe is isolated from the lysate and undergoes PvuII digestion followed by visualization on a 20% PAGE gel. The gel is quantitated using a Typhoon imaging system. The methyl group in the PvuII recognition sequence prevents cleavage in probes that have not been repaired. However, if MGMT repairs the oligo probe, removing the methyl group, then the PvuII can cleave the probe, producing a 10-bp oligo. Since it is known that 200 fmol of oligo were originally added, the fmol cleaved is calculated as follows: (intensity of 10-bp band)/(total intensity [10-bp band + 18-bp band])*200 fmol. Results are reported as fmol oligo cleaved/μg DNA. DNA is a more relevant normalization than protein for multiple reasons. First, DNA is the substrate for the MGMT activity. More importantly, immature and mature RMECs would be expected to contain the same amount of DNA, while protein content could vary widely between the two ages as a result of the different roles of RMECs at those two developmental stages.

While this is conceptually a very straightforward assay, its development and optimization for studying RMECs was not trivial. The first step was the production of the oligo. Single strand oligos were purchased and annealed, then diluted and aliquoted based on the amount of oligos reported on the certificate of analysis. The experimental meG oligo was produced first; later a control G oligo (same as the meG oligo, but lacking the methyl group) was produced to monitor PvuII cleavage efficiency. PAGE revealed a single band for each annealed oligo.

Because literature reports had indicated that RMECs would have low MGMT activity (5), the goal in initial experiments was to maximize the amount of input protein such that approximately half of the input oligo would be cleaved. Additionally, literature reports of the [^{3}H]methyl transfer assay reported using mg amounts of input lysate protein. The lysis preparation protocol was briefly as follows: Tissue (based on mass) or RMEC pellets (based on volume initially, but subsequently also based on mass) were resuspended in three volumes of MGMT assay buffer and homogenized on ice by three 30-second busts of a Polytron homogenizer. After sonication in five five-second bursts with 20-second rests, the lysate was centrifuged to pellet residue. Finally, the protein was concentrated using a Microcon 10 microconcentrator, which enriched the lysate in proteins greater than 10 kD. The BCA assay, which was already commonly run in the lab, was used to quantitate protein levels. Optimization experiments were initially performed using liver lysates, which were expected to have greater activity than RMECs and were much more readily available than the precious RMECs. In order to obtain sufficient amounts of protein, RMECs from multiple rats had to be pooled. Initially, single livers were used in the optimization experiments; however, in final reported assays, all lysates were produced using organs pooled from all animals from which RMECs had to be pooled.

The first optimization experiment examined the process of separating the oligo from the lysates following the initial incubation. A standard phenol:chloroform extraction was compared with a phenol:chloroform extraction facilitated by the use of phase-lock tubes. Sodium acetate and ammonium acetate were compared as the salts for the ethanol precipitation, and Qiaquick tubes eluted with 20 μL (the desired PvuII digestion volume) or 30 μL (the volume recommended by the instructions) were also tested. The Qiaquick tubes lost all oligo and were not further considered. It was determined that using phase-lock tubes followed by a sodium acetate-mediated ethanol precipitation was the procedure that retained the most oligo. A comparison of 1 vs. 2 phenol extractions indicated that the second extraction resulted in more oligo loss, while the addition of magnesium chloride in the ethanol precipitation facilitated greater oligo recovery.

The lysate production protocol was slightly modified. Following homogenization, the lysates were centrifuged to pellet residue and organelles, including the nuclei. The supernatant was stored on ice while the pellet was resuspended in one volume of assay buffer and sonicated thrice for 30 seconds with one minute rests. The supernatant previously removed was mixed back with the sonicate, and cellular debris was removed by centrifugation. After

microconcentration, the protein concentration was determined and the assay performed. These alterations were made in the hopes of increasing detectable MGMT activity.

It was noticed that residue which first appeared after microconcentration was present in the tubes following the phenol:chloroform extraction. An experiment was performed, and it was determined that this residue inhibited cleavage of the G (and presumably the repaired meG) oligo; thus, the residue was pelleted following the phenol:chloroform extraction and not carried to subsequent steps.

Throughout these initial experiments, there had been indication that there was possibly interference in the protein concentration determinations using the BCA assay. While the components of the buffer should not have been problematic, microconcentration may have created difficulties. Since literature reports utilized the Bradford assay for protein quantitation, that assay was adopted.

Because protein in RMEC lysates was much less concentrated than in other organs, larger volumes would be needed to achieve sufficient protein levels. Larger phase-lock tubes would be needed, requiring a different centrifuge, and, thus reoptimization. Because only one volume of isopropanol was required in contrast to three volumes of ethanol, the two were compared, with isopropanol being found ineffective in precipitating the oligo.

To prepare for using RMECs, an experiment was performed with liver extract at its most concentrated and diluted to the concentration expected for RMECs (with the same total protein in both conditions). The two concentrations gave different activity values and differed in oligo retention; this indicated a problem with the assay.

Another optimization experiment was performed. The protein concentration, oligo volume/concentration, presence of magnesium chloride during the oligo precipitation, microfuge tube size (1.5 vs. 2 mL), and alcohol (ethanol or isopropanol) were analyzed. Even using larger 2 mL (so fewer) tubes did not improve the efficiency of the isopropanol precipitation. Magnesium chloride made no difference with the ethanol precipitation, so was dropped from the protocol. Since the 1.5 mL tubes retained more oligo, they were used even when 2 mL tubes would result in less total tubes being needed. There were differences in cleavage again by concentration, indicating that cleavage was influenced by protein concentration.

Reconsideration of the literature specifically on this version of the assay (rather than the tritiated transfer version) indicated that the assay was performed on a much smaller scale, so the assay was optimized at that scale. It was found that a 150-μL reaction was successful. Residue was still found in the tubes when the DNA was precipitated and resuspended for PvuII digestion. An optimization experiment was performed, and it was determined that following the ethanol precipitation and resuspension in PvuII digestion buffer, the tubes should be centrifuged to pellet the residue. The supernatant containing the resuspended oligo was removed to another tube, where PvuII was added and digestion commenced. In another experiment, it was determined that the optimal PvuII digestion conditions were a 30 μL total reaction using 1.5 μL of PvuII; this gave the best G oligo cleavage without unspecific meG oligo cleavage.

Experiments to determine the viability of using lysates from NMU-treated rats were performed. Lysates from NMU-treated animals consistently showed a lack of MGMT activity. Experiments in which lysates from NMU- and control-treated rats were mixed exhibited MGMT activity around or lower than expected from purely mixing the activities. These results, both in liver and RMECs, indicate that something in the NMU-treated lysates inhibited the activity of the control-treated lysates. Likely, this effect is due to the presence of O^6-meG in the endogenous NMU-treated DNA. These adducts exhaust the MGMT activity in an unmeasurable way. In fact, the mixing experiments indicate that this contaminating DNA is also able to reduce activity in control lysates, presumably by acting as a substrate for MGMT. Additionally, the RMEC MGMT activity is already very low, approaching the limit of detection in control RMECs; the ability to detect further-diminished MGMT activity by this assay is questionable. For these empirically reinforced theoretical reasons, NMU-treated lysates will not be used in MGMT activity assays.

Dose-response experiments were performed to determine the optimal amount of protein to use in the assays. Experiments indicated that approximately 100 μg protein was the maximal amount to use; above this, the activity has plateaued and will not be an accurate representation of the enzymatic activity. Therefore, microconcentration, which added to the residue problem and increased the handling of the lysates, became unnecessary, and was dropped from the lysate

production protocol. Benzylguanine pretreatment of the lysates before oligo addition revealed the same amount of cleavage in the PvuII-digested and mock-digested lanes, which was the same as that seen under normal experimental conditions uncleaved by PvuII. Thus, cleavage found in non-PvuII-treated lanes was subtracted as background from the PvuII-cleaved lanes.

A Hoechst-based DNA assay was developed for lysate DNA quantitation. An experiment was performed to determine at what point in the lysate production to perform DNA analysis. Samples were assayed 1) following homogenization, 2) following homogenization and the first centrifugation, 3) following sonication when the supernatant was returned, and 4) in the final lysate. Protein concentrations remained relatively constant. Both centrifugation steps removed DNA from the lysate; thus, an aliquot was removed following homogenization but prior to the first centrifugation for DNA and protein analysis. The amount of protein used in each reaction was determined using the final lysate; that value was multiplied by the (DNA/protein) ratio from the removed aliquot (aliquot 1 above) to determine the DNA value to use for normalization. While the final oligo aliquots were below the limit of detection for this assay, concentrated stock oligos were assayed. They were rediluted and aliquoted based on the quantitation such that 5 μL contained 200 fmol.

Finally, experiments were performed in triplicate for liver and RMEC control lysates. Four experimental lanes (representing four separate reactions) were analyzed for each condition per experiment. In addition, two uncleaved lanes were run to determine background cleavage, and one lane with the G oligo was run to control for PvuII digestion. The means of the four experimental lanes (corrected for background) were averaged from three separate experiments (three separate sets of animals) and reported relative to DNA content (Figure 2).

KEY RESEARCH ACCOMPLISHMENTS

- The MGMT activity assay was optimized to a functional level.
- NMU-treated lysates cannot be measured using this assay methodology.
- RMECs have much lower MGMT activity than liver, regardless of age.
- MGMT activity is not age-dependent in liver.
- Immature RMECs exhibit statistically significantly lower MGMT activity than mature RMECs.

REPORTABLE OUTCOMES

The work reported here was the basis of an abstract submitted for the 2002 Era of Hope meeting to be held in September. It was also the basis of a platform presentation at the Fourth Annual Symposium on Developmental and Molecular Toxicology: Developmental and Genetic Aspects of Breast Cancer, held June 5-7, 2002, at the University of Wisconsin-Madison. Finally, results from this project served as preliminary data for a grant awarded by the Department of Defense to Michael N. Gould, DAMD 17-02-1-0624, "Mechanisms Underlying the High Risk for Breast Cancer Induction in the Immature Breast."

CONCLUSIONS

This reporting period has seen the successful optimization of the MGMT activity assay. Work from the last reporting period suggested age-related differences in MGMT activity; this was confirmed using the newly developed MGMT activity assay. MGMT activity in RMECs, which are targets of NMU-induced carcinogenesis was approximately 18-fold lower than in the liver, where NMU does not induce carcinomas (Figure 2). While liver (Figure 3) did not demonstrate any age-related differences in MGMT activity, immature RMECs exhibited statistically significantly lower activity than mature RMECs (Figure 2, Figure 4). This is consistent with the data obtained in the previous reporting period that demonstrated greater levels of DNA strand breaks in immature RMECs subsequent to NMU administration. These DNA strand breaks likely represent attempts at repair by base excision repair or mismatch repair, on which the immature RMECs must rely, due to their MGMT deficiency. It is consistent, then, that the immature RMECs, which had to rely on their lower fidelity DNA repair mechanisms, exhibit greater levels of persistent mutations (Big Blue data) that can contribute to increased susceptibility to mammary carcinogenesis.

So what? The work reported here clearly demonstrates that immature RMECs are more susceptible than mature RMECs to the mutagenic effects of NMU, due to their relative deficiency

in MGMT activity. It is known that the immature human breast is more susceptible than the mature human breast to the carcinogenic effects of ionizing radiation. The results here suggest that it would be prudent to investigate age-related DNA repair differences in human breast cells (and other tissues, as well). MGMT, the activity of which is induced by ionizing radiation (6, 7), would be a good first target, but other pathways, namely double strand break repair in the case of ionizing radiation, should be examined. Such basic knowledge of a process as crucial as DNA repair – and its developmental regulation -- would likely find applications beyond the understanding of carcinogenic initiation and the provision of novel targets for chemoprevention, for example in normal growth and development or other disease processes. More broadly, though, these results suggest that epidemiological studies seeking environmental chemicals responsible for breast cancer initiation should examine exposures of cohorts much younger than the adults already studied; studies of adult exposures should focus on promotion, rather than initiation. The ideal, though nontrivial, epidemiological study would be large, long-term, and prospective; following girls preferably from birth through puberty, and into adulthood. Exposures of concern would include known alkylating agents or their precursors encountered through the diet, for example in processed meat, or cigarette smoke (8-10).

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APPENDIX 1, FIGURES

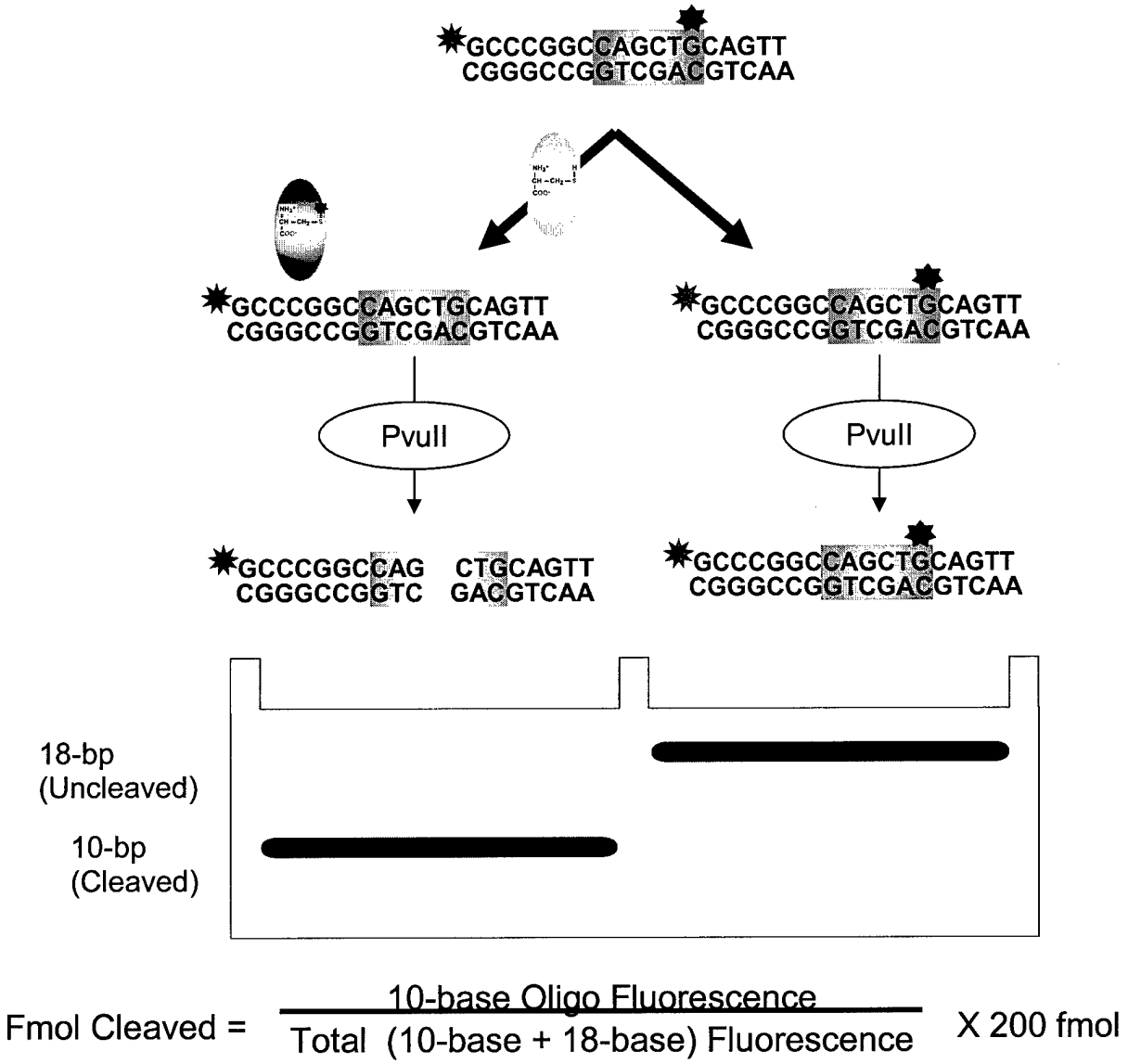

Figure 1. MGMT Activity Assay Schematic. The 18-bp meG oligo is shown at the top. The G oligo is the same except that it lacks the methyl group (star over the G at position 13). The star at the 5' position represents the hex label. In each reaction, the appropriate oligo (200 fmol in 5 μL) is mixed with 145 μL (approximately 100 μg) of protein. MGMT (vertical oval) present in the lysate repairs the oligo in a stoichiometric suicide fashion, as shown on the left. After two hours at 37° C, the protein is removed by phenol:chloroform extraction, followed by sodium acetate-mediated ethanol precipitation. The DNA is resuspended and transferred to a new tube, where PvuII restriction digestion is performed for two hours at 37° C. Repaired oligos are cleaved, as shown on the left. Unrepaired oligos cannot be cleaved, as shown on the right. The oligos are electrophoresed in a 20% denaturing PAGE gel, and the hex label is detected on a Typhoon imager. The amount of oligo cleaved is calculated as shown. This value is normalized by DNA content.

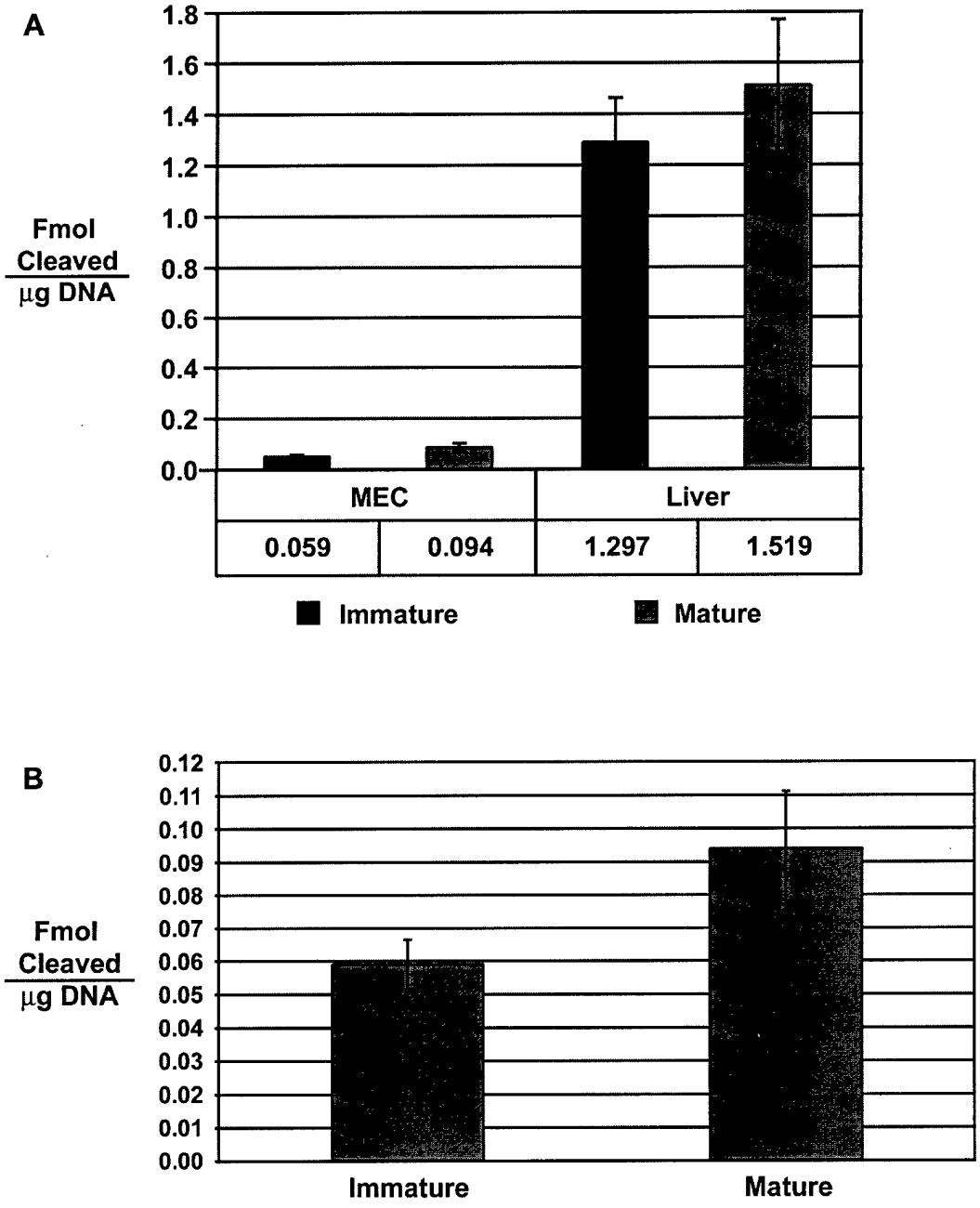

Figure 2. Methylguanine Methyltransferase Activity. Error bars represent the standard deviation. **A. Liver and RMEC MGMT Activity.** Liver MGMT activity is approximately 18-fold RMEC MGMT activity. **B. RMEC MGMT Activity** Immature RMECs have only ~ 3/5 the MGMT activity of mature RMECs. P=0.0127 Statistical significance was determined by pairwise one-tailed Student's t test.

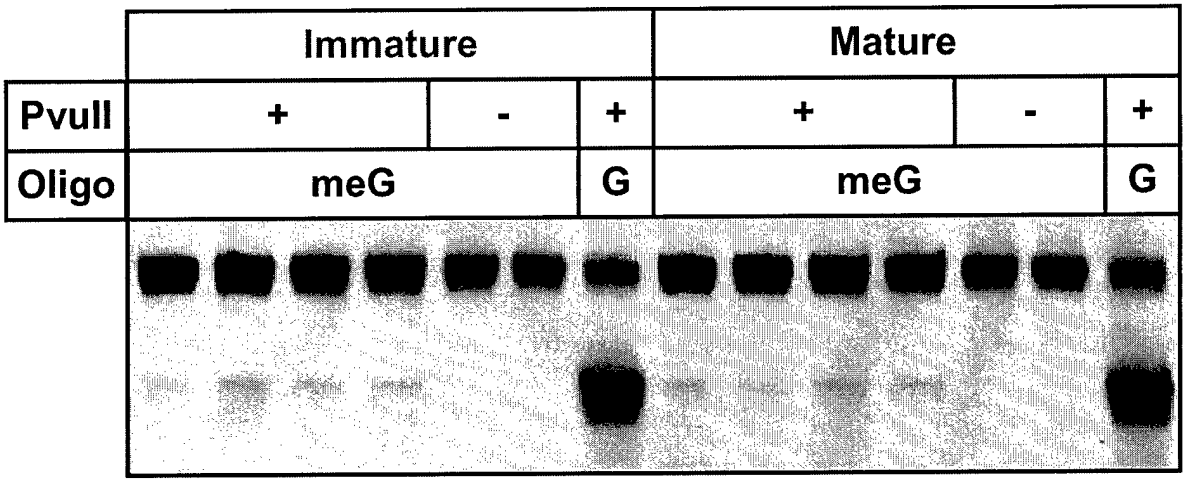

Figure 3. Liver MGMT Activity Assay Gel. The assay was performed using lysates with equivalent amounts of DNA. The first four lanes of each age show PvuII-cleaved meG oligo, and the next two lanes show uncleaved meG oligo. The final lane shows PvuII-cleaved guanine oligo. There is no age-related difference in MGMT activity in the liver.

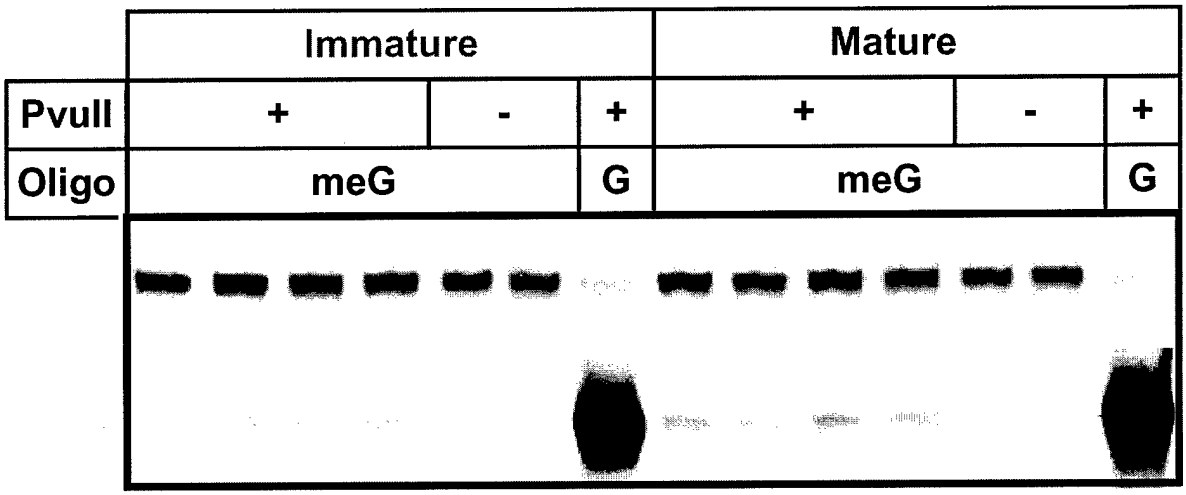

Figure 4. RMEC MGMT Activity Assay Gel. The assay was performed using lysates with equivalent amounts of DNA. The first four lanes of each age show PvuII-cleaved meG oligo, and the next two lanes show uncleaved meG oligo. The final lane shows PvuII-cleaved guanine oligo. While quantitation was performed with a uniform scan, the lower portion of this figure has been enhanced to make the 10-bp oligos visible. The scans are uniform horizontally across the gel. Immature RMECs exhibit significantly lower MGMT activity than mature RMECs.